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Asymmetric dimethylarginine confers the communication between endothelial and smooth muscle cells and leads to VSMC migration through p38 and ERK1/2 signaling cascade

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ABSTRACT

Communication between endothelial and smooth muscle cells (SMCs) contributes to atherosclerosis induced by atherogenic factors, such as oxide LDL. Asymmetric dimethylarginine (ADMA), a newly found cardiovascular risk factor, accumulates in the culture medium of oxide LDL (oxLDL)-treated endothelial cells and positively correlates with atherosclerosis. This study demonstrates that ADMA mediates the communication between endothelial cells and SMCs induced by oxLDL leading to SMC migration. In addition, the present study suggests exogenous ADMA directly induces SMC migration via p38 and ERK1/2 MAPK signaling transduction way. Investigations to identify the factors regulating VSMC migration may provide novel insights into atherosclerosis and its complications.

1. Introduction

Atherosclerosis and its complications, including myocardial infarction, stroke, and peripheral vascular disease, remain a major cause of death in industrialized countries [1]. Vascular smooth muscle cell (VSMC) migration plays a critical role in the initiation and progression of intimal thickening in atherosclerotic lesions [2]. Interactions between endothelial cells and VSMCs are involved in both normal functions of the vessel wall and the development of atherosclerosis. In this context, communication between endothelial cells and VSMCs has to be taken into account when investigating the migration of VSMCs into the arterial wall.

Oxidized low-density lipoprotein (oxLDL) contributes to VSMC migration; however, the mechanisms responsible for the effects of oxLDL on VSMC migration are not yet fully understood. Recently, asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS) and a newly found cardiovascular risk factor, was reported to accumulate in the culture medium of oxLDL-treated endothelial cells [3]. However, whether ADMA mediates the communication between endothelial and smooth muscle cells leading to VSMC migration remains uncertain.

We hypothesized that ADMA mediates the communication between endothelial and smooth muscle cells that is triggered by oxLDL and leads to VSMC migration. In addition, since ADMA induces mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase1/2 (ERK1/2), c-Jun NH2-terminal kinase (JNK), and p38MAPK, which are a family of central signaling molecules that respond to numerous stimuli in vascular smooth muscle cells [4], we also hypothesized that the impact of ADMA on VSMC migration would be partly due to ADMA-induced activation of MAPK signal transduction.

Investigations to identify factors regulating VSMC migration may provide novel insights into atherosclerosis and its complications.

2. Materials and methods

2.1. Antibodies and major reagents

See Supplementary material.

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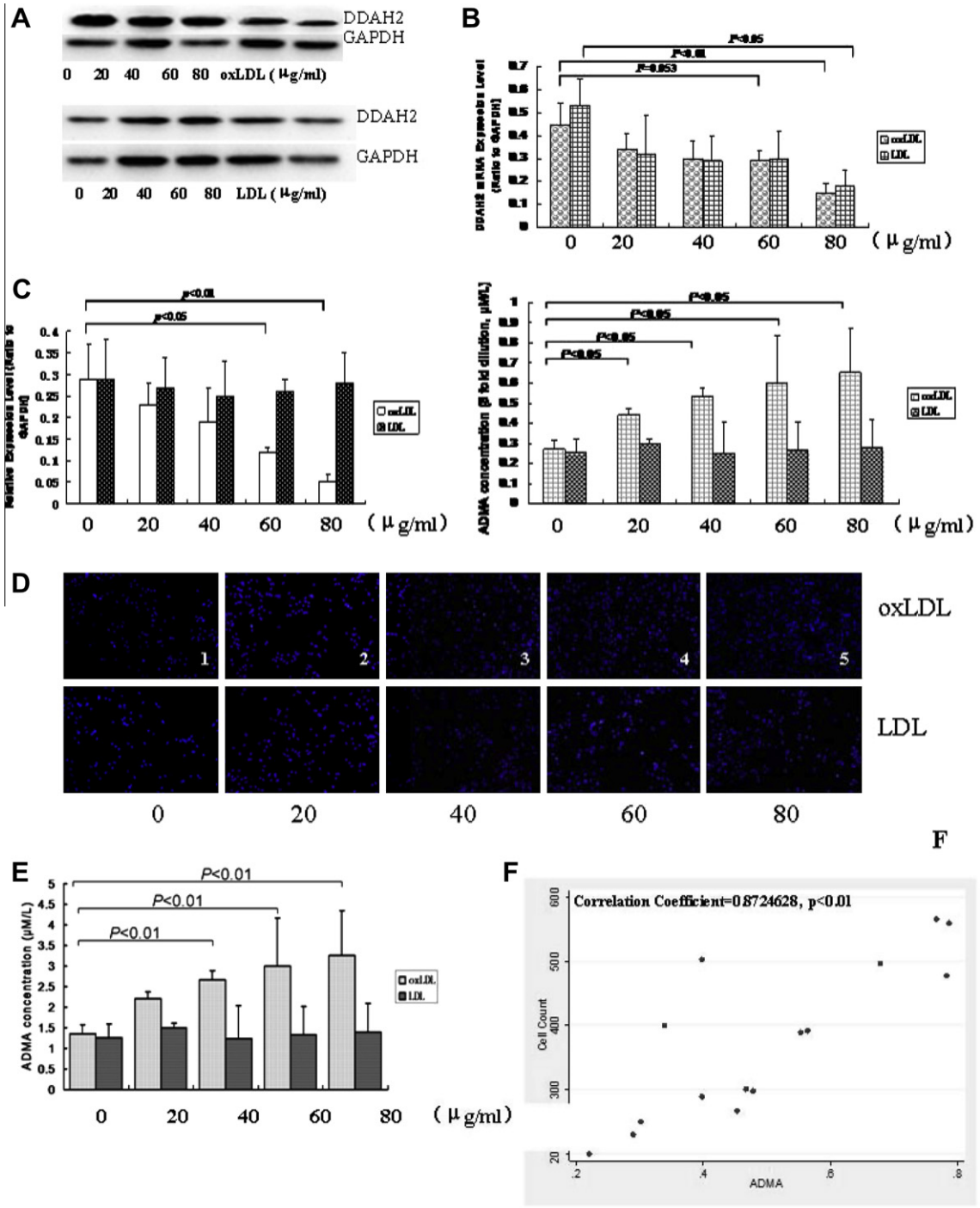

Fig. 1. HUVEC-conditioned medium pretreated with oxLDL induces VSMC migration. HUVECs were pretreated with oxLDL at different concentrations (0, 20, 40, 60, and 80 μg/ml) for 12 h. Native LDL was used as a control. The supernatant was collected and used as conditioned medium. In the Transwell migration assay, VSMCs were seeded in the upper chamber of the Transwell and the conditioned medium was supplemented in the bottom chamber. The growth medium was DMEM (without L-arginine) containing 5 mM hydroxyurea to prevent HUVEC and VSMC proliferation. Twelve hours later, cells that migrated through the 8 μm pores were stained with 4′,6-diamidino-2-phenylindole · 2HCl (DAPI). The number of VSMCs that migrated onto the lower surface of each filter was counted in different fields at a magnification of 200× by three independent observers with Image Pro Plus 5.0 software (Media Cybernetics, GA, US). (A–B) DDAH2 protein and mRNA expression level in HUVECs. (C) ADMA concentration in the conditioned medium of HUVECs. D: Representative images of VSMCs that migrated through the pores of a Transwell chamber. Images 1–5 are representative of treatment with HUVEC-conditioned medium pretreated with oxLDL or LDL at the concentrations of 0, 20, 40, 60, and 80 μg/ml, respectively. E: The correlation between ADMA concentration in the conditioned medium and the number of migrated VSMCs. Data, expressed as mean ± S.E.M., are representative of at least three independent experiments.

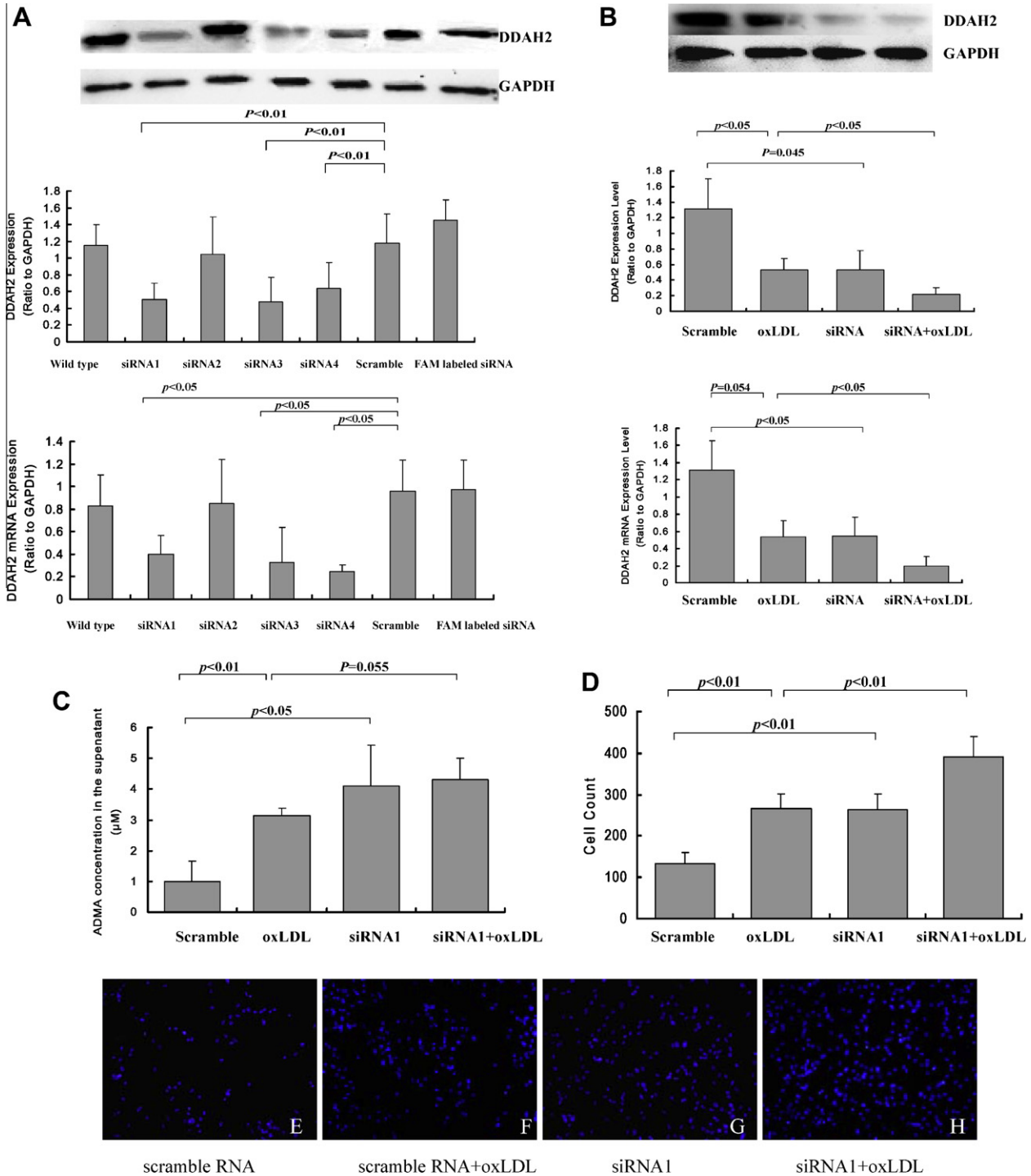

Fig. 2. *DDAH2* knock-down enhances the oxLDL-mediated induction of VSMC migration. The following siRNAs were generated against the human DDAH2 mRNA sequence: siRNA-1, 5′-UGCUGACAGAUCACCCAUATT-3′; siRNA-2, 5′-GCUCCGAAUUGUGGAAAUATT-3′; siRNA-3, 5′-GAUCUGGCCAAAGCUCAAATT-3′; and siRNA-4, 5′-GCUGACAGAUCACCCAUAUTT-3′. As controls, a scrambled siRNA and negative control siRNA not exhibiting homology to any coding region of *DDAH2* were used. The FAM labeled negative control siRNA was used for transfection control. Subsequently, siRNA-1 was selected for further use. After being transfected with siRNA-1 or scrambled siRNA for 24 h, HUVECs were treated with oxLDL (60 μg/ml). The growth medium was DMEM (without L-arginine) containing 5 mM hydroxyurea to prevent HUVEC proliferation. (A) DDAH2 protein and mRNA expression levels in HUVECs transfected with different siRNAs. (B) DDAH2 protein and mRNA expression level in different (Transfected with DDAH2 siRNA or not) treated with or without oxLDL (60 μg/ml). (C) ADMA concentration in HUVEC-conditioned medium. (D) Number of VSMCs that migrated through the pores of a Transwell chamber after treatment with HUVEC-conditioned medium. (E–H) Representative images of VSMCs that migrated after stimulation with different HUVEC-conditioned medium. Data are expressed as mean ± S.E.M. and are representative of at least three independent experiments.

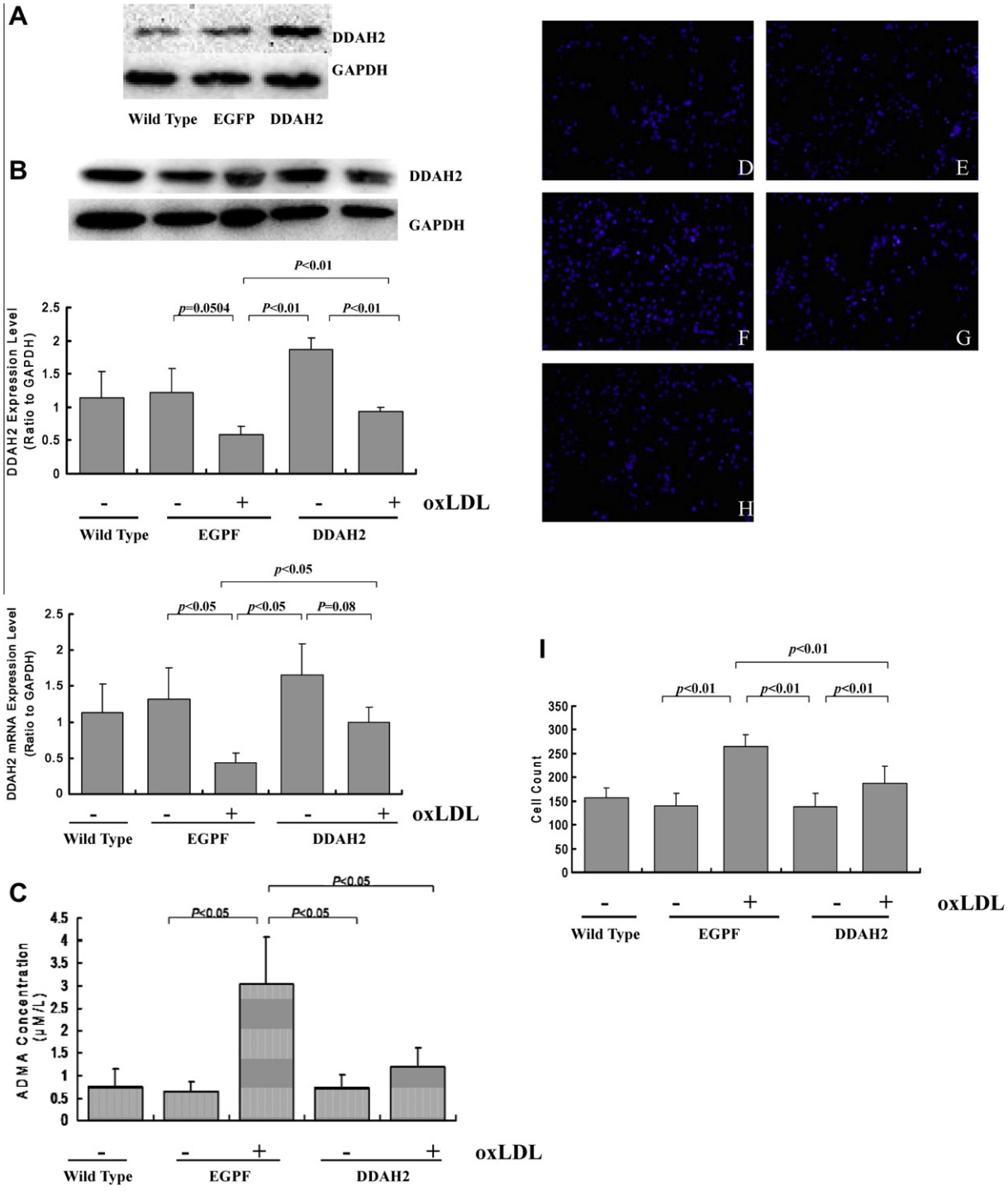

Fig. 3. Overexpression of *DDAH2* in HUVECs prevented the induction of VSMC migration by HUVEC-conditioned medium. hDDAH2 expression vector or the empty EGFP vector were transiently transfected into HUVECs, and the HUVECs were treated with oxLDL (60 μg/ml). (A) DDAH2 protein expression level in HUVECs pretreated with different transfections. (B) DDAH2 protein and mRNA expression levels in HUVECs treated with or without oxLDL. (C) ADMA concentration in HUVEC-conditioned medium. (D–H) Number of migrated VSMCs and representative images of VSMCs in Transwell assay after treatment with HUVEC-conditioned medium. Data are expressed as mean ± S.E.M. and are representative of at least three independent experiments.

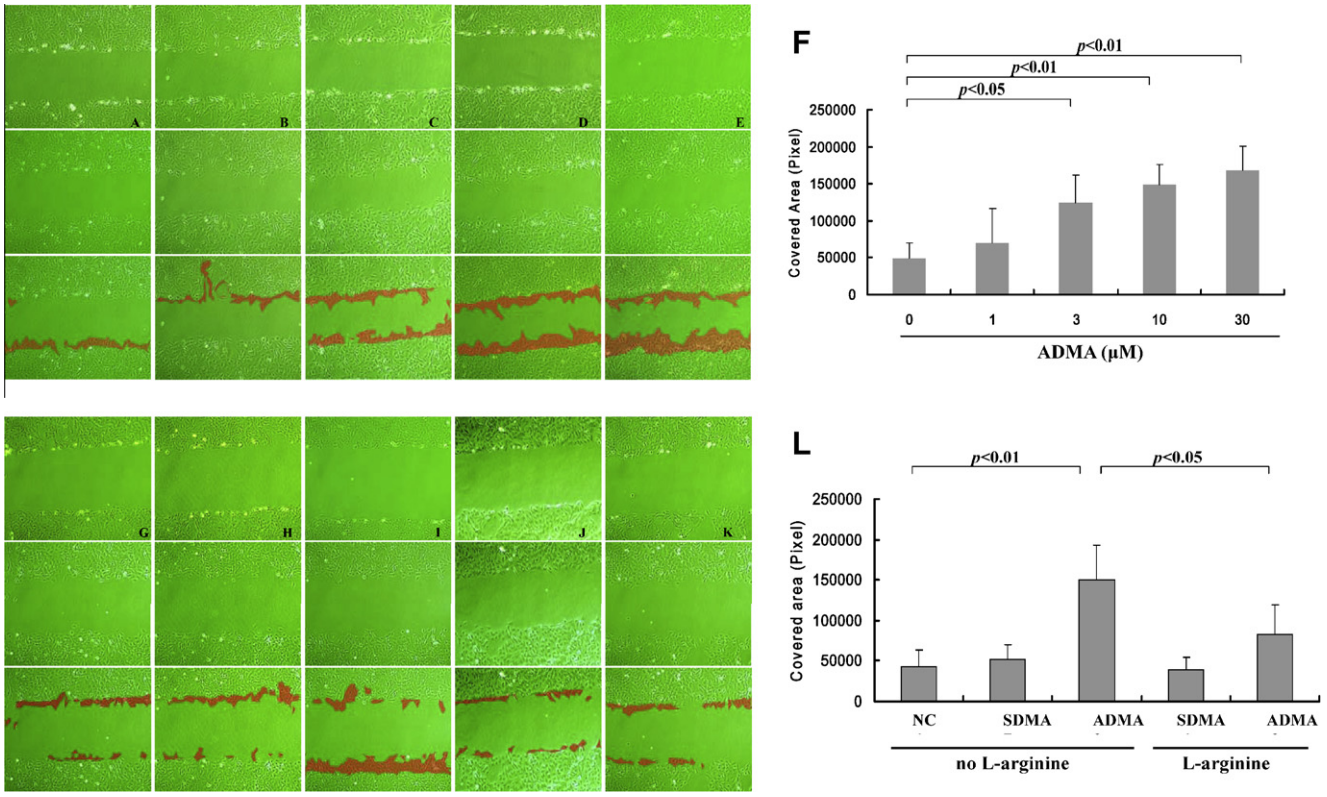

Fig. 4. Exogenous ADMA induces VSMC migration. Confluent VSMCs (starved for 48 h in FCS-free DMEM) were treated with ADMA at different concentrations (0–30 μM) for 24 h. The migration activity was expressed as the change in covered area. (A–E) VSMC migration induced by 0, 1, 3, 10, and 30 μM ADMA for 24 h, respectively. The upper, middle, and bottom images represent the beginning point, end point, and merged images, respectively. The red shadow represents the change in covered area. Samples were run in triplicate in three independent experiments. (F) Change in covered area.

2.2. Preparation of LDL and oxLDL

LDL was isolated from healthy blood as previously described [5] and oxidation of LDL was performed by dialyzing against 10 μM $CuSO_4$ in PBS for 24 h at 37 °C as described [6]. (Detailed protocol was included in Supplementary material.)

2.3. Cell culture

See Supplementary material.

2.4. DDAH2 overexpression and knock-down

ADMA is hydrolyzed to L-citrulline and dimethylamine by dimethylarginine dimethylaminohydrolase 2 (DDAH2) in the cardiovascular system. The strategies of DDAH2 overexpression and knock-down were used to modulate the level of ADMA. The protocol was attached in the Supplementary material.

2.5. VSMC migration assay

Tanswell Assay was used to evaluate VSMC migrating ability [7]. Detailed protocol was enclosed in Supplementary material.

2.6. Wound-healing assay

Cell migration was also assessed using wound-healing assays as previous described [8]. The migration/growth medium was DMEM without L-arginine containing 5 mM hydroxyurea to prevent VSMC proliferation. Four different fields of migration were photographed with a video camera system using Image Pro Plus 5.1 software at the intersection of the previously marked line and wound edge before and after treatment with ADMA. The migration activity was expressed as the change in covered area.

2.7. Western blotting

Immunoprecipitation and immunoblotting were performed as previously described [9].

2.8. Determination of ADMA concentration

The level of ADMA in the conditioned medium was measured by ELISA (DLD Diagnostika, Germany). Detailed protocol was included in Supplemental material.

2.9. Statistical analysis

Data are expressed as mean + S.E.M. Each experiment was repeated three times, and representative results are shown. The Kolmogorov–Smirnov method was used to test the normal distribution [10]. One-way ANOVA was used to analyze values, followed by Student's *t*-tests to distinguish significant differences. A P-value < 0.05 was considered significant.

3. Results and discussion

3.1. oxLDL induces ADMA accumulation in HUVEC-conditioned medium via the inhibition of DDAH2 activity and expression

In agreement with previous studies [11], the results presented here show that oxLDL markedly increased ADMA accumulation via the inhibition of DDAH2 activity and the reduction of DDAH2 expression (Fig. 1A and B) in HUVECs.

DDAH2, the main subtype of dimethylarginine dimethylaminohydrolase in the cardiovascular system, is the specific hydrolase of ADMA and modulates ADMA levels. DDAH2 activity is modulated at the post-translational and transcriptional levels, which be directly inactivated by various oxidative or inflammatory stimuli. The expression level can also be down-regulated by lipopolysaccharide (LPS) or high glucose [12]. In this study, the concentrations of oxLDL required to have an effect on DDAH2 activity and DDAH2 expression are different. oxLDL treatment between 20 and 80 μg/ml led to a significant decrease in DDAH2 activity that was represented by the accumulation of ADMA (Fig. 1C). When HUVECs were treated with oxLDL at a concentration exceeding 60 μg/ml, the expression of DDAH2 at both the mRNA and protein levels was markedly decreased (Fig. 1A and B). Thus, oxLDL induces ADMA accumulation in HUVEC-conditioned medium via the inhibition of DDAH2 activity and expression.

3.2. ADMA facilitates communication between HUVECs and VSMCs and leads to VSMC migration

Cell communication can occur via soluble mediators. Mediators secreted by endothelial cells can lead to VSMC migration, collagen synthesis, VSMC phenotype change, and secretion of hyaluronic acid. In previous studies [13,14], ADMA was induced by Hcy and Ang II in endothelial cells and led to the apoptosis of VSMC. In this study, the conditioned medium from oxLDL-treated HUVECs markedly increased the number of VSMCs that migrated through the Transwell chamber (Fig. 1D and E), indicating that a soluble factor was generated in endothelial cells and secreted into the medium. Considering the accumulation of ADMA in the conditioned medium, we proposed that ADMA was the critical mediator that induced VSMC migration. In fact, the positive correlation between ADMA concentration in the medium and the number of migrated VSMC supports the conclusion that ADMA is an interaction factor between endothelial cells and VSMCs (correlation coefficient = 0.8724628, $P < 0.01$, Fig. 1F).

To further confirm the role of the DDAH2/ADMA pathway, the strategies of DDAH2 knock-down and overexpression were applied. Transfection of HUVECs with DDAH2 siRNA successfully knocked down DDAH2 expression at both the mRNA and protein levels in oxLDL-treated and untreated HUVECs (Fig. 2A and B). In contrast, scramble siRNA, not targeting any known cDNA, did not affect DDAH2 expression. Our results showed that the effect of oxLDL on the accumulation of ADMA in HUVEC medium was amplified by DDAH2 knock-down (Fig. 2C) and the inducing effects of HUVEC-conditioned medium on VSMC migration was significantly

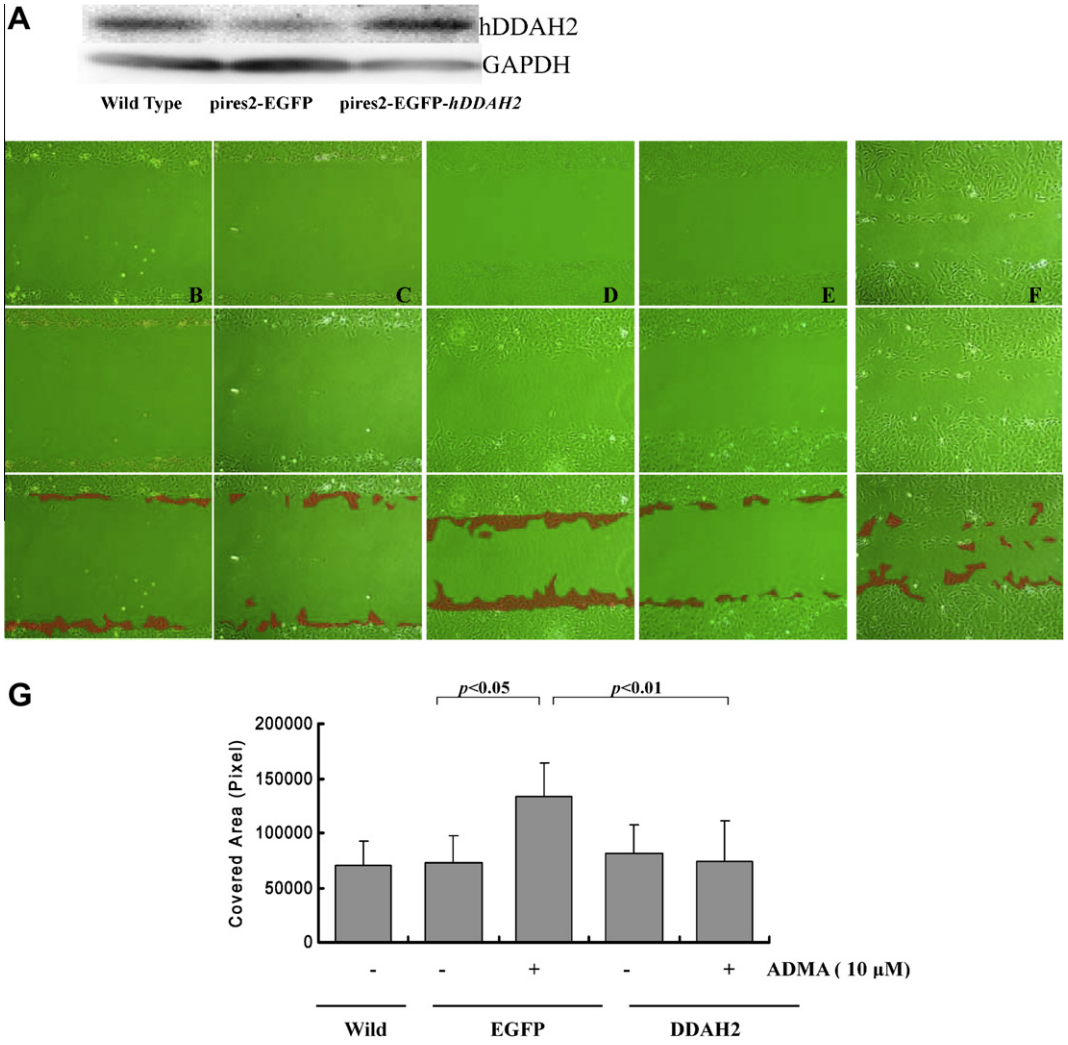

Fig. 5. DDAH2 blunts the inducing effects of ADMA on VSMC migration. VSMCs transfected with the hDDAH2 expression vector or the empty EGFP vector were treated with ADMA (10 μM) for 24 h. All transfections were carried out in triplicate, and each experiment was reproduced four to six times. The migration activity was expressed as the change in covered area. (A) DDAH2 expression level in VSMCs with different transfections. (B–F) VSMC migration with different treatments. The upper, middle, and bottom images represent the beginning point, end point, and merged images, respectively. The red shadow represents the change in covered area. (F) Change in covered area.

enhanced by DDAH2 knock-down as shown by the increase in the number of migrated cells (Fig. 2D–H).

We constructed a human DDAH2 expression vector and transiently transfected it into HUVECs (Fig. 3A and B). DDAH2 overexpression in HUVECs blunted the accumulation of ADMA in the conditioned medium (Fig. 3C) and reduced the number of VSMCs that migrated (Fig. 3D–I). The empty pIRES2-EGFP vector did not influence ADMA generation or VSMC migration.

Our results indicate that overexpression of DDAH2 significantly blunted the accumulation of ADMA in the conditioned medium and reduced the number of migrated VSMCs. On the contrary, knock-down of *DDAH2* enhanced the elevation of ADMA in the supernatant and increased the number of migrated VSMCs. Our results demonstrate that ADMA facilitates the communication between endothelial and smooth muscle cells and finally leads to VSMC migration. This study is the first to report the role that the DDAH2/ADMA pathway plays in oxLDL-induced VSMC migration.

3.3. Exogenous ADMA directly induces VSMC migration

To further confirm the contribution of ADMA in the process of oxLDL-induced VSMC migration, the effect of exogenous ADMA was observed in wild-type and hDDAH2-overexpressing VSMCs. ADMA induced VSMC migration in a concentration-dependent manner in the concentration range of 3–30 μM, as assessed by the confluent area of VSMCs in a wound-healing assay (Fig. 4A–E). According to the previously reported highest plasma ADMA levels in patients with different clinical conditions [15] and in experimental atherosclerotic animals [16,17] and the popularly used ADMA concentrations for in vitro studies, 10 μM ADMA was selected for further investigation in this study.

Then the same concentrations of symmetric dimethylarginine (SDMA), an ADMA analogue, were used as controls in order to investigate whether the inducing effects on VSMC migration were ADMA-specific. Treatment of VSMCs with 10 μM ADMA increased VSMC migration 2.9-fold (Fig. 4I and L, $P < 0.01$), while SDMA did not (Fig. 4H and L, $P > 0.05$), indicating that the effect on VSMC is ADMA-specific. To distinguish whether the effects of ADMA on VSMC migration is NO-dependent, we used DMEM (without L-arginine) as the growth medium [18]. Therefore, the effect of ADMA on VSMC migration is NO-independent. It is interesting that the precursor of NO, L-arginine, markedly blocked ADMA-induced VSMC migration (Fig. 4J–L), indicating that supplemental NO donors can partly blunt the pathological effects of ADMA on VSMC. Because ADMA is a specific substrate of DDAH2, transient transfection of the hDDAH2 expression vector into VSMCs (Fig. 5A) significantly attenuated the migration induced by exogenous ADMA (Fig. 5B–G). Our results confirmed that the DDAH2/ADMA pathway plays an important role in VSMC migration.

Pretreatment with L-arginine and overexpression of hDDAH2 attenuated the activation of VSMC migration induced by ADMA, suggesting that supplemental NO donors or acceleration of ADMA degradation can blunt the pathological effect of ADMA on VSMCs. This is in agreement with previous studies [19–21]. ADMA mediates multiple pathological effects, including apoptosis, oxidative and nitrodative stress, proliferation, and pro-inflammation in VSMCs [22–24]. Our demonstration of the effect of ADMA on VSMC migration may have implications on future studies of artherosclerosis. The effect of DDAH2 overexpression or L-arginine on VSMC migration indicates potential therapeutic strategies for artherosclerosis.

3.4. ADMA induces VSMC migration via the p38MAPK and ERK1/2 signal transduction pathways

VSMC migration may be involved in multiple signaling pathways [25]. MAPKs, ERK1/2, JNK, and p38MAPK, are a family of central signaling molecules that respond to numerous stimuli and participate in cell proliferation and migration [26]. Activation of JNK, p38MAPK, and ERK1/2 induces migration, and inhibition of JNK, p38MAPK, and ERK1/2 protects VSMCs against migration [26,27].

In this study, ADMA significantly activated the ERK1/2 and p38MAPK signaling transduction pathways, but not the JNK signal transduction pathway in VSMCs (Supplementary Fig. 1). To determine whether the ERK1/2 and p38MAPK pathways were involved in ADMA-induced migration of VSMCs, PD98059 (ERK1/2-specific inhibitor) and SB203580 (p38MAPK-specific inhibitor) were used. Pretreatment with specific inhibitor of p38MAPK and ERK1/2 (SB203580 and PD98059) significantly inhibited phosphorylation of p38MAPK and ERK1/2 (Supplementary Fig. 2) and attenuated ADMA-induced cell migration (Supplementary Fig. 3), indicating that their activation contributed to ADMA-induced migration of VSMCs.

4. Conclusion

In conclusion, oxLDL induces the accumulation of the soluble factor ADMA in HUVECs via the inhibition of DDAH2 activity and expression, thereby inducing VSMC migration. Also, ADMA induces VSMC migration via the p38MAPK and ERK1/2 signal transduction pathways. To the best of our knowledge, this is the first study to demonstrate cell communication facilitated by ADMA that leads to VSMC migration. We hope that this study contributes novel insights regarding potential therapy for and prevention of atherosclerosis and its complications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.07.032.

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